Volume 215, number 1, 151–154 FEB 04634 May 1987

Iron binding to microsomes and liposomes in relation to lipid peroxidation

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Received 24 February 1987

The effects of ADP, ATP, citrate and EDTA on iron-dependent microsomal and liposomal lipid peroxidation, and on $^{59}FeCl_3$ binding to the lipid membranes were measured. The aim was to test if initiation of lipid peroxidation is a site-specific mechanism requiring bound iron. In the absence of chelator, iron was bound to both membranes. EDTA and citrate removed the iron and inhibited peroxidation. ATP and ADP stimulated peroxidation, but whereas ADP allowed only half of the iron to remain bound, all was removed by ATP. Chelators, therefore, cannot be simply influencing a site-specific mechanism. Their effects must relate to the reactivities of the different iron chelates as initiators of lipid peroxidation.

Lipid peroxidation; Microsome; Iron; Chelator

1. INTRODUCTION

Liposomal and microsomal lipids are susceptible to iron-dependent peroxidation, although the mechanism of the process is not well understood [1–3]. With dispersed lipid the reaction can be catalysed by Fe(EDTA) with requirements for a reducing agent and H_2O_2, implying that the mechanism involves free hydroxyl radicals ($OH^{\cdot}$) [4–6]. However, peroxidation of lipids within membranes is inhibited by EDTA, does not generally require H_2O_2, and is not inhibited by hydroxyl radical scavengers [4,6,7]. Some chelators, such as ADP and ATP, stimulate microsomal or liposomal peroxidation [1,8,9]. The ways in which chelators influence the reaction are not fully understood, but one possibility is that they alter the site of generation of oxidants. It has been proposed, mainly to explain differences between dispersed and membrane lipids, that like other damaging radical reactions [10], initiation (The term initiation is used throughout to describe the initial event in the chain of lipid peroxidation in its broadest sense. This could be either a lipid hydroperoxide independent (hydrogen abstraction) or dependent reaction [3].) of membrane peroxidation may be site localized [2,3,11]. It would occur where iron is associated with the membrane, and involve a short-lived species reacting at this site before diffusing into the bulk phase. Such a site may not be accessible to scavengers that would otherwise inhibit the reaction. Negatively charged phospholipid groups are possible binding sites.

According to this mechanism, iron should be bound to the membrane in the absence of chelator, or in the presence of the weaker chelators ADP or ATP, but not in the presence of EDTA. Such a proposal has not been directly tested. We, therefore, have measured iron binding to liposomes prepared from ox brain phospholipids, and to rat liver microsomes, and examined the effects of ATP, ADP, citrate and EDTA. Parallel measurements of iron-dependent lipid peroxidation, induced either by hypoxanthine and xanthine oxidase or by NADPH, have been made.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

Liposomes were prepared from phospholipid extracted from ox brain, shaken at 5 mg/ml in 0.15 M NaCl [12]. Microsomes were prepared from the livers of Sprague Dawley rats [13]. Microsomal protein was determined according to Lowry et al. [14]. All procedures were carried out in plastic or acid-washed glassware, and buffers were treated with chelex resin (BioRad, CA) to minimize iron contamination. Reactions were all carried out in 10 mM phosphate buffer, pH 7.4. Biochemicals were from Sigma. Xanthine oxidase (type I) was treated to remove EDTA and contaminant iron [16]. $^{59}FeCl_3$ (spec. act. 2–40 mCi/mg) was from NEN.

Liposomes (0.75 mg/ml) were incubated with hypoxanthine (150 μM), xanthine oxidase (approx. 0.01 U/ml) and 1 μM $FeCl_3$ premixed with the required chelator. The enzyme concentration was adjusted to give an initial superoxide (O_2^-) generation rate of 3 μM/min, measured separately as the rate of cytochrome *c* reduction. Microsomes (approx. 0.41 mg protein/ml, giving a rate of cytochrome *c* reduction of 3 μM/min) were incubated with 1 μM $FeCl_3$ premixed with chelator as required, and 100 μM NADPH. Reactions were carried out at 22°C for 45 min, with continuous mixing by rotation. Lipid peroxidation was measured as thiobarbituric acid (TBA) reactivity, as described in [15].

Liposomes (0.75 mg in 1 ml) or microsomes (0.41 mg protein in 1 ml) were incubated with 1 μM $FeCl_3$ (containing approx. 1.3×10^4 dpm/ml of ^{59}Fe), premixed with the requisite chelator, for 30 min at 22°C. After ultracentrifugation at 105000 × *g* for 1 h, free and bound iron was measured by analysing the supernatant and pellet, respectively, for radioactivity. Residual supernatant counts in the pellet amounted to approx. 1% of the total.

3. RESULTS

As expected from previous studies [1,2], iron-dependent peroxidation occurred with either liposomes and a hypoxanthine/xanthine oxidase O_2^--generating system, or with microsomes and NADPH (fig.1). The effects of chelators were examined with 1 μM added iron (fig.2). With both systems, ATP and ADP enhanced lipid peroxidation, EDTA fully inhibited, and citrate substantially decreased the reaction. Maximum effect was seen with 0.25 mM EDTA, but higher concentrations of the other chelators were required. Breakdown of endogenous lipid peroxides contributed little to the TBA reactivity. Liposomes or microsomes alone heated with Fe or Fe(ATP) gave < 10% of the A_{532} values obtained for enzymatic peroxidation under the same conditions.

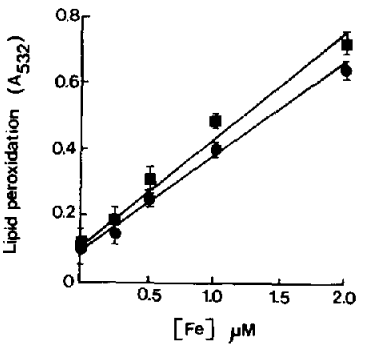

Fig.1. Dependence of liposomal (■) and microsomal (●) lipid peroxidation on iron concentration. Reaction conditions were as described in section 2 with the final concentrations of $FeCl_3$ as shown. Each point is the mean ± SD for two sets of duplicates.

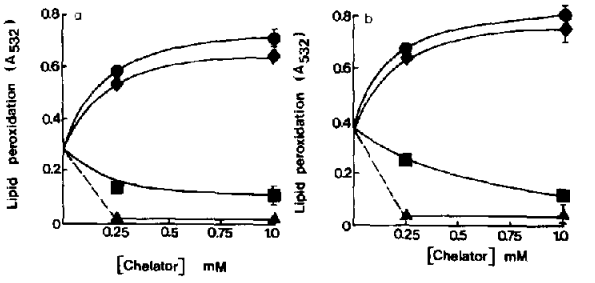

Fig.2. Effects of chelators on (a) liposomal and (b) microsomal lipid peroxidation. Reactions were carried out as described in section 2 with 1 μM $FeCl_3$ and ATP (●); ADP (◆); citrate (■) and EDTA (▲). Each point is the mean ± SD for two or three sets of duplicates. No error bars are shown where the SD is within the symbol height.

To ensure that the effects of the chelators in the liposomal system were not due to their removing iron bound to the xanthine oxidase [16], peroxidation of liposomes induced by adriamycin (30 μM) ferredoxin reductase (which has a lower affinity for iron) and NADPH was studied. Chelator ef-

fects similar to those in fig.2a were seen (not shown).

In the absence of chelator, with a total of 1 μM Fe in solution, approx. 50% was bound to 0.75 mg/ml liposomes and 45% to 0.41 mg/ml microsomes. Iron binding increased with increasing liposome or microsome concentration (not shown). It was prevented by EDTA (fig.3). The low residual counts in the EDTA pellets are accountable for by the trapped supernatant. ATP and citrate also removed most of the iron from the liposomes, but with 1 mM ADP, approx. 50% was still bound (fig.3a). This was also the case with the microsomes, except 1 mM ATP or citrate allowed slightly more iron to remain bound (fig.3b).

With 10 μM iron in solution, approx. 7.0 nmol were bound/mg liposomes, and 6.0 nmol/mg microsome protein. This was removed by 100 μM EDTA, but in the presence of 1 mM ADP, 78 ± 3 and 75 ± 7% of the iron (means and ranges of two assays) remained bound to the liposomes and microsomes, respectively. Hence slightly more iron at the higher concentration remained bound. It is not possible to distinguish whether this was free iron not removed by ADP, or iron-ADP complexes associated with the membranes.

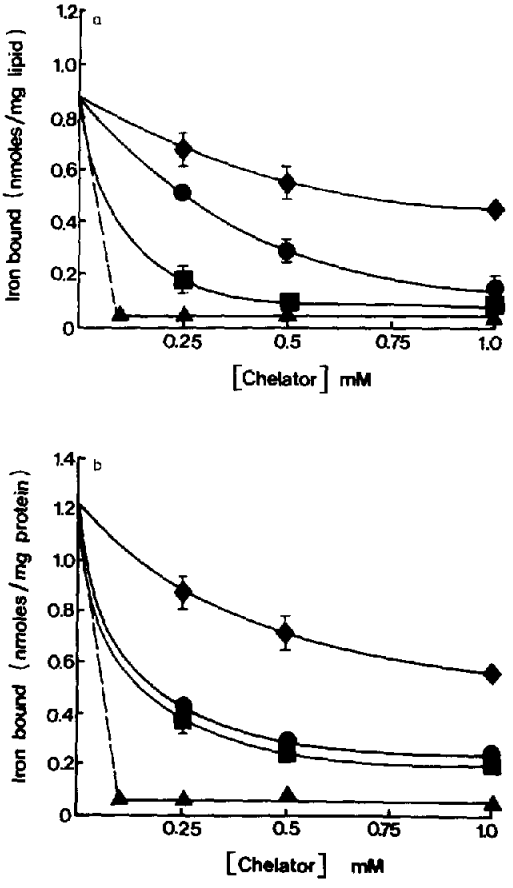

Fig.3. Effects of chelators on ^{59}Fe binding to (a) liposomes and (b) microsomes. Reactions were carried out as described in section 2 with ATP (●), ADP (♦), citrate (■) and EDTA (▲) at the concentrations shown. Each point is the mean ± SD for three or four measurements. No error bars are shown where the SD is within the symbol height.

4. DISCUSSION

We have shown that when lipid peroxidation occurred in the absence of chelator, a substantial proportion of the iron present was bound, either to liposomes or microsomes. EDTA and citrate inhibited both processes in parallel. These results would be compatible with membrane-bound iron initiating lipid peroxidation. However, although ATP and ADP both gave a concentration-dependent increase in lipid peroxidation, appreciable iron was bound only in the presence of ADP. Hence, even if an argument can be made for oxidant production from bound iron in the presence of ADP, with ATP, the iron species responsible for lipid peroxidation must have been generated in solution, and not at a site on the liposomes or microsomes.

Since a site-specific mechanism does not explain why iron catalyses peroxidation in the presence of some chelators but not others, it is likely that their influence is on the properties of the oxidant responsible. Our observed lack of lipid peroxidation with Fe(EDTA), which is an excellent catalyst of $OH^{\cdot}$ production from $O_2^{\cdot-}$ and H_2O_2 [1,2,17], and in the microsomal system [15], supports others [1,4,6,7,19] who have concluded that $OH^{\cdot}$ is not the initiating species. Although Fe(ADP) and Fe(ATP) can react with H_2O_2 to give $OH^{\cdot}$ [20], each is $<5\%$ as efficient as Fe(EDTA) at catalysing the Haber-Weiss reaction [18,21]. Catalysis of lipid peroxidation by these complexes also points to an oxidant other than $OH^{\cdot}$. Recent studies suggest that nonchelated Fe^{2+} and H_2O_2 react to produce an Fe(IV) species [22], but if it participated in lipid peroxidation, a requirement for H_2O_2 would

be expected. Several studies have implicated a perferryl or $Fe^{3+}O_2^-$ species [1,8], and others have shown that microsomal peroxidation is optimal when both Fe^{3+} and Fe^{2+} are present [9,23]. It may be that ATP or ADP facilitates the formation of an iron-oxygen complex, perhaps perferryl, and as originally proposed by Hochstein et al. [8], this initiates peroxidation.

One qualification must be placed on our results. Lipid peroxidation requires cycling between Fe^{3+} and Fe^{2+}, and it is possible that Fe^{2+} (or any iron-oxygen complex) could have different binding characteristics from those of Fe^{3+}. These would have to be achieved, however, within the time of each catalytic cycle.

We conclude that although site-specific generation of the iron species responsible for initiating lipid peroxidation is not excluded by our findings, the effects of chelators on the process cannot be explained strictly in these terms. The difference in reactivity of the iron chelates must be a major determinant.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council of New Zealand, and the Cancer Society of New Zealand.

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